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Molecular dissection of the cauliflower mosaic virus translation transactivator

Marc De Tapia[1], Axel Himmelbach and Thomas Hohn[2]

Friedrich Miescher Institute, Basel, Switzerland
[1]Present address: UPR 9002 du CNRS, Institut de Biologie Moléculaire et Cellulaire, 15 rue Descartes, 67084 Strasbourg Cedex, France
[2]Corresponding author

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The cauliflower mosaic virus (CaMV) transactivator (TAV) is a complex protein that appears to be involved in many aspects of the virus life cycle. One of its roles is to control translation from the polycistronic CaMV 35S RNA. Here we report a molecular dissection of TAV in relation to its ability to enhance dicistronic translation in transient expression experiments. We have identified a protein domain that is responsible and sufficient for that activity. This 'MiniTAV domain' consists of only 140 of the 520 amino acids in the full-length sequence. A further domain located outside the MiniTAV, and therefore dispensable for transactivation, is probably involved in interactions with other molecules. This was identified by its ability to compete with wild-type TAV and some of its deletion mutants. We found, furthermore, that the TAV protein binds RNA. Two regions needed for RNA-binding properties were defined outside the MiniTAV domain and RNA binding seems not to be directly involved in the transactivation mechanism.

Key words: caulimovirus/pararetrovirus/RNA binding/transactivation/translational control

Introduction

Cauliflower mosaic virus (CaMV; Figure 1A) is the type member of caulimoviruses, a group of pararetroviruses infecting plants. While CaMV transcription is quasi-constitutive, its translation is strongly controlled. One reason for this might be the alternative use of the CaMV 35S RNA as a replicative intermediate or as mRNA for at least six coding regions (Mason *et al.*, 1987; Hohn and Fütterer, 1991). Two different mechanisms seem to be involved in translational control:

(i) A 600 nt leader with six or seven small open reading frames (sORFs), depending on the strain used, constitutes *a priori* a strong barrier to translation of the first larger ORF (ORF VII). Nevertheless, circumvention of this hurdle is astonishingly efficient, seems to be host controlled and depends on specific sequences within the leader (Fütterer *et al.*, 1989; Fütterer and Hohn, 1993). According to the shunt model (Fütterer *et al.*, 1990b), the 'scanning unit', which was assumed to be the 40S ribosome plus initiation factors (Kozak, 1989) or a set of initiation factors alone (Sonenberg, 1991; Thomas *et al.*, 1992), initiates scanning at or near the cap site as usual (Kozak, 1989). However, after a certain distance it is transferred from a position 5′ of the leader to a 3′ position by a mechanism so far unknown in detail.

(ii) A second, unusual mechanism is required for translation of the five cistrons located further downstream on the 35S RNA, which are silent in *in vitro* translation systems (Gordon *et al.*, 1988) both in the absence and the presence of CaMV ORF VI (here called transactivator, TAV) but are active in its presence in protoplast transient expression systems (Bonneville *et al.*, 1989) and transgenic plants (Zijlstra and Hohn, 1992). ORF VI is the only CaMV ORF for which an individual mRNA, the monocistronic 19S RNA, has been found (Guilley *et al.*, 1982). The second cistron of a whole range of model dicistronic mRNAs was translated in several types of transfected plant protoplasts when TAV was co-expressed (Bonneville *et al.*, 1989). Specific *cis* signals seem not to be required (Fütterer and Hohn, 1991). In fact, transactivation works on dicistronic RNAs that totally lack CaMV sequences, although certain

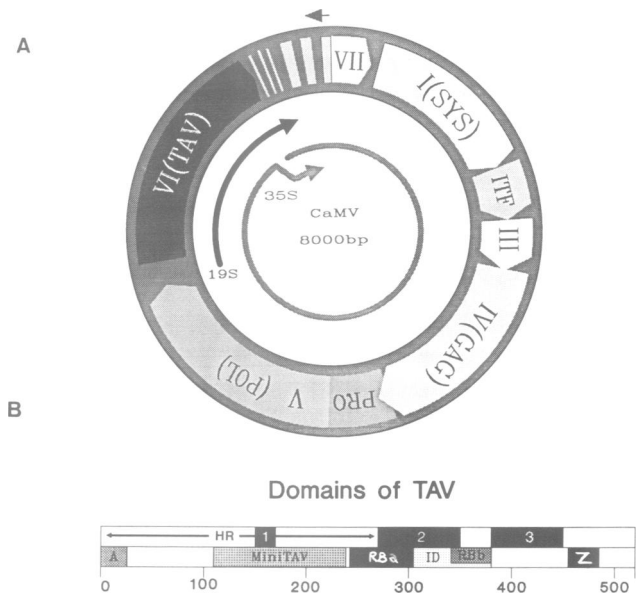

Fig. 1. CaMV maps. (**A**) Circular map of CaMV DNA showing the origin of the minus strand (arrow on the top), the arrangement of the ORFs [VII (no known function), I (SYS, systemic spreading), II (ITF, insect transmission factor), III (no known function), IV (GAG, polyprotein, structural proteins), V (enzymatic functions; PRO, protease; POL, polymerase + RNase H), VI (TAV, transactivator)], six unassigned small ORFs within the leader, and the RNAs. TAV is translated from the 19S RNA; the other ORFs are translated from the polycistronic 35S RNA under control of TAV. (**B**) Linear map showing properties of the the ORF VI product (TAV protein). The first row shows regions of very strong (1, 2) and strong (3) sequence preservation within the caulimovirus group, as well as the variable region responsible for host range (HR; interrupted by homology region 1); the second row shows the sequence with features of a signal peptide (A), the minimal region required for transactivation action (MiniTAV), the putative RNA-binding domains (RBa and RBb), the zinc-finger-like sequence (Z) and the interactive domains (ID) proposed from the competition experiments.

structural features influence the degree of transactivation, e.g. an sORF in front of the first cistron strongly enhanced transactivated translation of the second cistron, and strong secondary structure at the 5′ end of the RNA inhibited translation of all cistrons (Fütterer and Hohn, 1991, 1992). These and other findings suggest that the transactivation mechanism involves translation machinery that starts scanning at the cap site and that translation of the downstream cistron is coupled to translation of one or more of the upstream ORFs. A similar mechanism of transactivation was reported for the related figwort mosaic virus (FMV) (Gowda *et al.*, 1989; Scholthof *et al.*, 1992), and the transactivators of CaMV and FMV are interchangeable in transient expression experiments. A transactivation mechanism also exists in the yeast GCN4 translational system and is induced by starvation (for a review see Abastado *et al.*, 1991). Upstream ORFs also modulate the transactivation of translation of a downstream coding region in this case, but only short and simple upstream ORFs are tolerated, in contrast to the large ORFs containing many internal AUGs that can constitute the first cistron in the CaMV system.

In addition to controlling translation, the TAV protein has other functions important for the virus life cycle, that may be connected indirectly to the transactivation function. It was first described as the main component of the virus inclusion bodies (Covey and Hull, 1981), large membrane-free structures with the size of organelles (Shepherd, 1976; Rodriguez *et al.*, 1988). These are the sites of viral DNA synthesis (Pfeiffer and Hohn, 1983; Bonneville *et al.*, 1984; Mazzolini *et al.*, 1985), the accumulation of all viral gene products including those of non-viral passenger genes incorporated into the virus genome (deZoeten *et al.*, 1989), and of assembly (Shepherd, 1976). They are surrounded by ribosomes (Shepherd, 1976) at an early stage. Inclusion bodies may also be a storage form of virus particles, inhibiting reinfection of the nucleus. Such a regulative function has been assigned to the *env* protein of HBV (Summers *et al.*, 1990). The TAV protein is also responsible for the host range and for the severity of symptoms (Daubert *et al.*, 1984; Schoelz and Shepherd, 1988; Stratford and Covey, 1989; Daubert and Routh, 1990). Non-host plants (Baughman *et al.*, 1988; Kiernan *et al.*, 1989; Takahashi *et al.*, 1989; Balàsz, 1990; Goldberg *et al.*, 1991) and the host plant *Arabidopsis thaliana* (Zijlstra and Hohn, 1992) transgenic for the TAV coding region under the control of its own 19S promoter or under the more efficient 35S promoter, show symptoms, the severity of which may be related to the expression level of the transgene. Infectivity experiments using CaMV with a hybrid TAV showed that its N-terminal region is responsible for the host range specificity (Daubert *et al.*, 1984; Schoelz *et al.*, 1986).

The TAV protein (Figure 1B) has a length of 520 amino acids and a mobility on SDS−PAGE gels corresponding to 60 kDa. Conservation of the caulimovirus TAV varies in different protein regions of FMV (Richins *et al.*, 1987; Sanger *et al.*, 1991), carnation edge ring virus (CERV) (Hull *et al.*, 1986), and soybean chlorotic mottle virus (SoyCMV) (Hasegawa *et al.*, 1989). In general, the C-terminal half is more conserved, especially between amino acids 283 and 350 ('caulimoblock'), whereas the N-terminal half is more variable, reflecting its role in host adaptation. The first 26 amino acids show sequence features of a signal peptide (Blobel and Dobberstein, 1975), and the stretch between residues 455 and 480 is very rich in cysteine and histidine residues, which in CaMV are in an arrangement ($FHCX_3CHFX_{13}HHC$) resembling zinc fingers (Evans and Hollenberg, 1988).

This paper describes an analysis of the TAV protein as a step towards understanding the transactivation mechanism. We present evidence that the TAV protein is a mosaic of different domains which function independently and have different properties.

Results

Transactivation by TAV and its deletion derivatives

Nicotiana plumbaginifolia protoplasts were cotransfected with combinations (5 μg each) of (i) pHELP7, the standard plasmid expressing TAV or a derivative of it, (ii) the dicistronic reporter plasmid pCAT2, which in the presence of TAV expresses chloramphenicol acetyl transferase (CAT) and (iii) the monocistronic reporter plasmid pMonoGUS, which expresses β-glucuronidase (GUS) independently of TAV. The latter was used to normalize the results. Confirming the results of Bonneville *et al.* (1989), the complete system expressed CAT and GUS, while the system lacking pHELP7 expressed only GUS. Obviously, CAT expression from the dicistronic plasmid depended on the presence of TAV.

CAT yields were not reduced when pHELP7 was replaced by a helper plasmid lacking either the first 27 or the first 111 codons of the TAV ORF (pDel-1 and -2; see Materials and methods) (Figure 2), while plasmids lacking the first 183 codons (pDel-3) did not promote CAT expression. The proportion of the TAV C-terminus which could be removed without abolishing CAT activity was even larger; truncation of the TAV ORF by up to 278 codons, i.e. up to residue 242 (pDel-4, -5, -6 and -7), still allowed CAT expression, albeit at a reduced level (Figure 2). In these cases, the DNAs and mRNAs were not shortened but the TAV ORF was truncated by insertion of a 26 nt linker containing stop codons in all three phases. Therefore, the alteration in transactivation is very likely a property of the translation product rather than of the nucleic acid or its transcription and translation. The same results were obtained with shorter plasmid versions where sequences downstream of the inserted stop codon were removed (results not shown), showing that read-through of the stop codon cannot be responsible for the residual TAV activity. A reduced level of CAT activity was also found for internal TAV deletions covering the region 242−378 (pDel-9, -10 and -11); however, transactivation was no longer observed when the TAV ORF was truncated up to residue 214 (pDel-8) or internally deleted between residues 214 and 295 (pDel-12).

These results show that TAV regions between amino acids 112−183 (pDel-2 versus pDel-3) and 215−241 (pDel-7 versus pDel-8) contain essential parts of the TAV protein. We therefore constructed the plasmid pMiniTAV, which combines the deletions of pDel-2 and pDel-7 and codes for a polypeptide encompassing TAV residues 112−241. This plasmid was also active in the transactivation assay (Figure 2).

CAT activities indirectly represent TAV activities and since equal amounts of TAV or TAV derivative DNA were transfected in our experiments, the degree of saturation of transactivation was not known. To approach this concept of TAV activities more accurately, we compared the degree

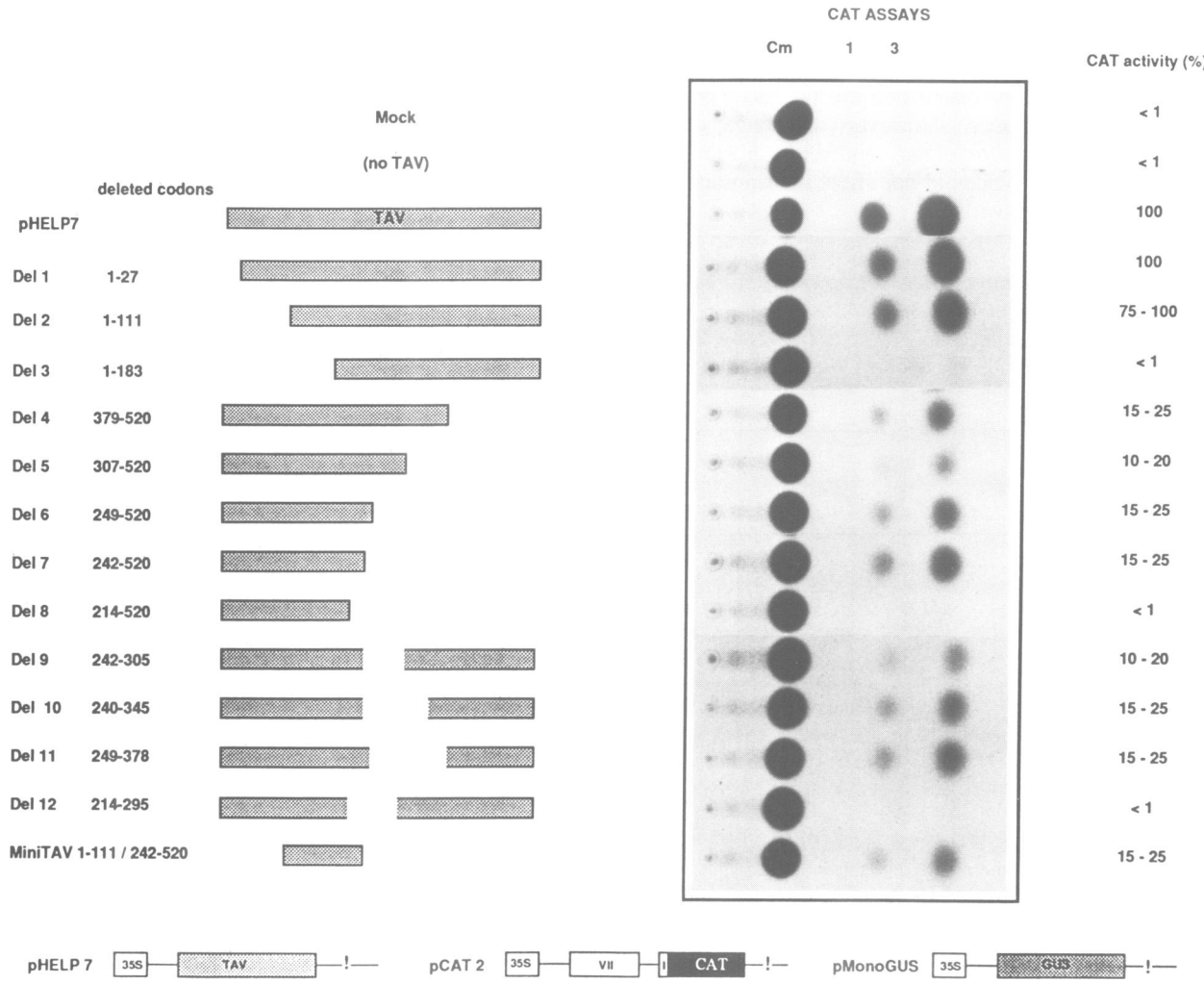

Fig. 2. Transactivation of dicistronic translation by TAV and a series of deletion mutants. The transactivator plasmid pHELP7, the dicistronic reporter plasmid pCAT2 and the monocistronic reporter control plasmid pMonoGUS used in standard transactivation experiments in PEG-transfected protoplasts from *N.plumbaginifolia* leaves are shown at the bottom of the figure. The main figure shows the name of the deletion mutant (first column), codons deleted (second column), schematic representations of the TAV ORF and its deletion mutants (third column), typical CAT assays (fourth column) and CAT activities as measured in at least three independent experiments (fifth column).

of saturation of each combination and analyzed the activity of TAV or representative members of our mutant series as a function of the amount of transactivator plasmid in our transfection experiments (Figure 3). Two different types of curve were obtained. With pHELP7, pDel-2 and pMiniTAV, CAT activities reached 100% saturation levels, albeit at different transactivator plasmid concentrations; with pDel-7 and pDel-11, CAT activities only reached ~20% and 30% of that level, respectively. Comparison of the 50% saturation points showed that the apparent specific activity of TAV was ~10 times higher than that of Del-2, ~100 times higher than that of MiniTAV and ~50 times higher than that of Del-7 and Del-11. Plasmids pDel-2 and pMiniTAV produced RNAs with the same leader sequence and AUG context and, with the exception of the 26 nt linker sequence, the same length. This suggests that differences between the apparent transactivation activities are due to differences in protein properties rather than different levels of transcription and translation. The reason for the lower saturation levels of Del-7 and Del-11 is not known, but might, for example, be caused by the inhibitory action of an improperly folded subpopulation.

Analysis of the putative active center of TAV

Since the MiniTAV protein is still able to act as a transactivator, it can be assumed that the active center of TAV is located within the 140 amino acids comprising this polypeptide. Surprisingly, this region slightly overlaps the more variable part of TAV that determines the virus host range. However, a computer alignment of the ORF VI amino acid sequences in CaMV, CERV (Hull *et al.*, 1986) and FMV (Richins *et al.*, 1987) revealed that this part also contains an island of high homology (amino acids 139–158; Figure 4A). The corresponding ORF of SoyCMV (Hasegawa *et al.*, 1989) initially appeared to lack this sequence motif but was found when we inspected the −1 reading frame; we assume that it was misplaced by a sequencing (frameshift) error and belongs in fact to the ORF VI coding sequence. The motif is an arrangement of aromatic and hydrophobic amino acid residues around a strictly conserved structure-breaking GP dipeptide (Figure 4B).

Computer analysis of the motif predicts a β-sheet/turn/β-sheet structure. The tryptophan (W) residue at the end of the block, which is also strictly conserved, should be located already outside of the second β-sheet, since it always follows

at least one acidic residue. We mutagenized the motif in the context of the full-length TAV construct to test its importance in transactivation. Deletion of the central YNGP tetrapeptide (Figure 4B) completely inactivated the transactivator and indicated its importance. However, exchange of the conserved W residue for either related (F, Y or H) or unrelated (Q) amino acids did not affect the transactivator capacity (data not shown).

Defective TAV molecules that act as competitive inhibitors of functional TAV

The second half of the TAV protein (aa 242–520) is dispensable, although it contributes strongly to the full activity of TAV. Its contribution could be at the general level of protein folding, structure and integrity, but it may also be involved in protein–protein or protein–RNA interactions, thereby supporting the indispensable MiniTAV domain. To characterize such interactive domains we set up an assay to test defective TAV mutants for their ability to compete with active TAV. If an inactive TAV mutant still contains domain(s) able to interact with part of the transactivation complex (e.g. active transactivator, ribosomes, host cellular factors or RNA), a reduction of transactivation (reporter gene expression) would be expected.

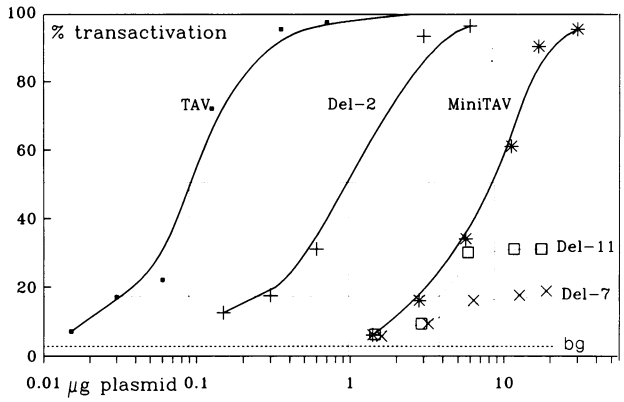

Fig. 3. Transactivation capacity of TAV and TAV derivatives. Dependence of CAT activities on the concentration of plasmids expressing TAV and TAV derivatives in standard transactivation experiments as in Figure 2. bg, background.

In our competition assays, four plasmids were co-transfected into the protoplasts: the dicistronic reporter plasmid pBIGUS (5 μg), the monocistronic reporter plasmid pMonoCAT (0.5 μg), the transactivator plasmid (at a sub-saturation quantity of 0.2 μg) and the presumptive competitor plasmid in increasing amounts (0.2–10 μg). All these plasmids were under the control of the same (35S) promoter. GUS expression provides an indirect measure of transactivation levels, while CAT expression provides a measure of transfection efficiencies. Such an internal standard is necessary since transfection efficiencies can vary from sample to sample, even when derived from the same protoplast batch. CAT expression was not affected by addition of either transactivator or competitor even in large quantities (up to 40 μg DNA), and we conclude that neither of them affects general transcription and translation rates.

The presence of competitor plasmid pDel-12 markedly reduced GUS expression from the dicistronic reporter plasmid pBiGUS (Figure 5), showing that the inactive TAV protein derived from pDel-12 does in fact competitively inhibit the TAV protein. We used truncated Del-12 derivatives expressed by plasmids pDel-12:4 and pDel-12:5

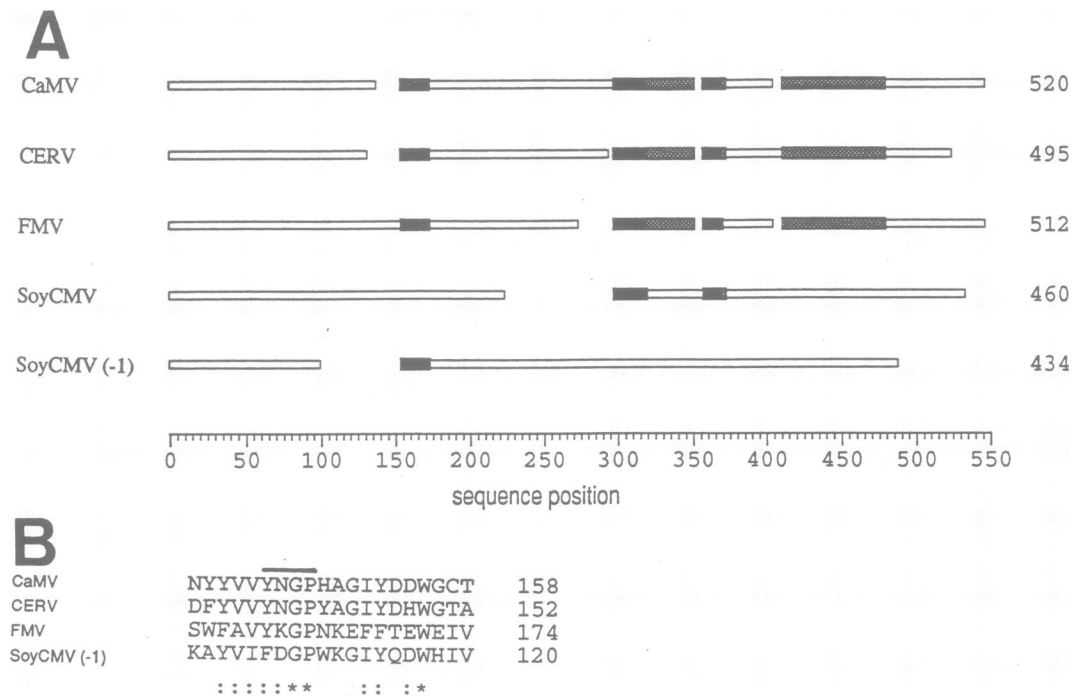

Fig. 4. Multiple alignment of CaMV coding regions. (**A**) The 'multiple alignment construction analysis workbench' (MACAW) computer program (Schuler *et al.*, 1991) was used. The designated ORF VI coding regions were analyzed for the caulimoviruses, but for SoyCMV all three reading phases were considered; the 0 and −1 phases, which revealed homology with the other viruses, are shown. The homologies with minimal MP scores of 38 found for all four viruses are shown by the darkest shading [18 codons, MP 53.7 (sequences shown at the bottom); 23 codons, MP 47.7; 16 codons, MP 38.7]; homologies found for only three of the viruses are shown by lighter shading (69 codons, MP 141.7; 69 codons, MP 46.0). (**B**) Sequences of the putative active center; conserved (*) and related (:) amino acids are marked. The central tetrapeptide is marked by a line.

to map the domain of the Del-12 protein implicated in this inhibitory effect. Del-12:4 protein competed as strongly as Del-12, while Del-12:5 did not compete significantly (Figure 5). This comparison shows that sequences between amino acid positions 307 and 378 are required for competition and can be regarded as a molecular interaction domain.

TAV functions in a wider range of cells than MiniTAV

In contrast to our results with *N.plumbaginifolia* protoplasts shown above, which confirmed the findings of Bonneville *et al.* (1989), none of our TAV mutants showed residual activity in *Orychophragmus violaceus* cell culture protoplasts, regardless of whether pBiGUS or pCAT2 was used as the dicistronic reporter plasmid and despite the fact that transactivation with full-length TAV was very efficient in these cells (saturation was obtained for *N.plumbaginifolia* with 0.5 μg of DNA per 6×10^5 protoplasts and for *O.violaceus* with 1.25 μg per 2×10^6 protoplasts). One explanation for this observation could be that the TAV mutants are for some reason not stable in *O.violaceus* cells and transactivation cannot be observed. In order to test this hypothesis, we used pDel derivatives as competitors of TAV expressed by pHELP7 in *O.violaceus* cell culture protoplasts. While neither of these plasmids themselves caused transactivation, they in fact inhibited transactivation by TAV (Figure 6). This result strongly suggests that the mutant proteins are indeed expressed in host cells, are not degraded and retain the interactive domains required for competition. Thus, either some functions present in *N.plumbaginifolia* mesophyll protoplasts can replace the support domains of TAV, or the action of the TAV mutants in question is inhibited by some factors present in *O.violaceus* cell culture protoplasts and the support domain in TAV alleviates this inhibition, or conditions within *O.violaceus* cells do not allow the proper folding of mutant TAV proteins.

Full-length TAV binds RNA through domains located outside of MiniTAV

Since transactivation acts at the translation level, we investigated the affinity of TAV for RNA. For this purpose, the TAV gene was expressed in *Escherichia coli* and TAV protein was isolated and used for *in vitro* studies. The cloning was perfomed using a pDS vector (Bujard *et al.*, 1987) that allows high levels of transcription under *lacZ* promoter control and high levels of translation due to a properly spaced Shine−Dalgarno sequence. As observed frequently for overexpressed proteins, the TAV protein produced within the bacterial cells formed dense and insoluble protein aggregates (bacterial inclusion bodies, BIB). Such aggregates can usually be easily and rapidly purified by differential centrifugation following cell lysis (Nagai and Thøgersen,

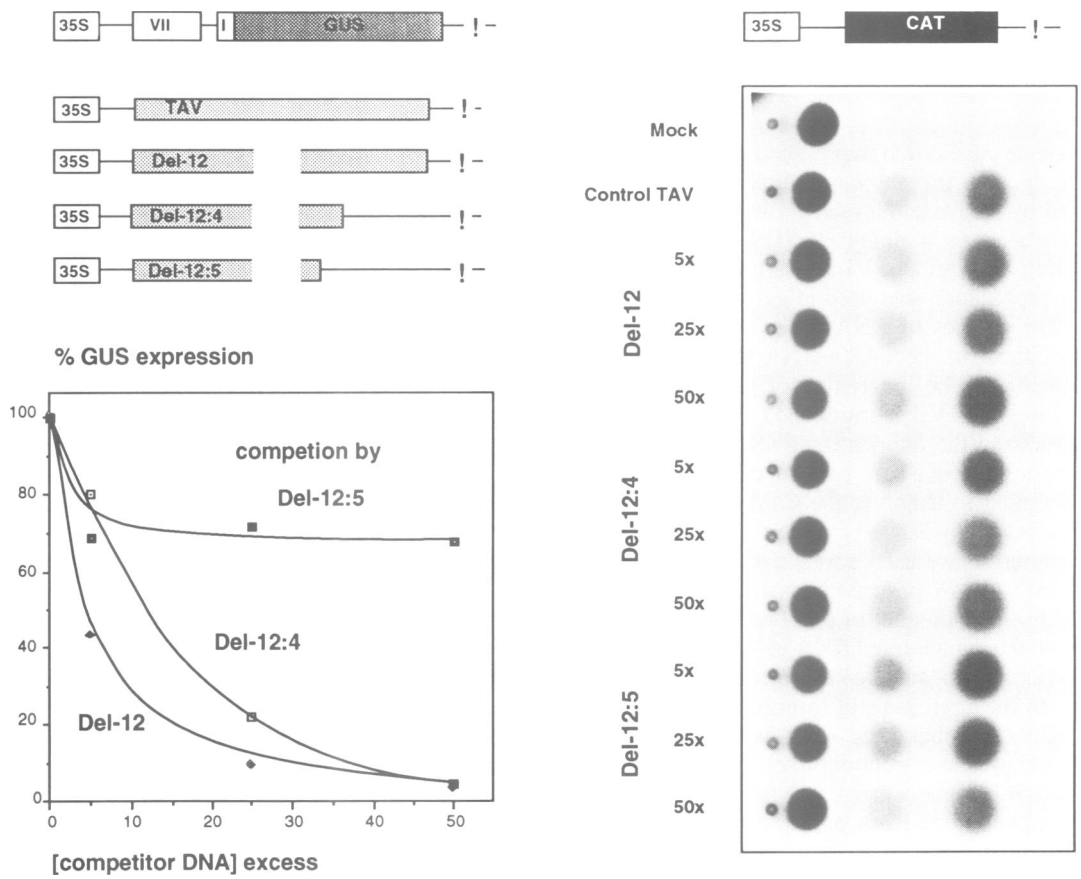

Fig. 5. Competition of wild-type TAV and derivatives by deletion mutants. The reporter plasmid pBiGUS, pHELP7 expressing wild-type TAV and competitor plasmids pDel-12, pDel-12:4 and pDel 12:5 (at 5 ×, 25 × and 50 × excess concentrations respectively compared with pHELP7) were PEG-cotransfected into *N.plumbaginifolia* leaf protoplasts. These plasmids are shown at the top of the figure. GUS expression resulting from each combination is represented as % of expression corrected by the internal control value. The 100% value corresponds to GUS expression obtained in the absence of competitor plasmid. CAT activities derived from the internal standard (pCAT3, shown on the top of the CAT assays) were not affected by pHELP7 and the competitor plasmids as shown on the right panel.

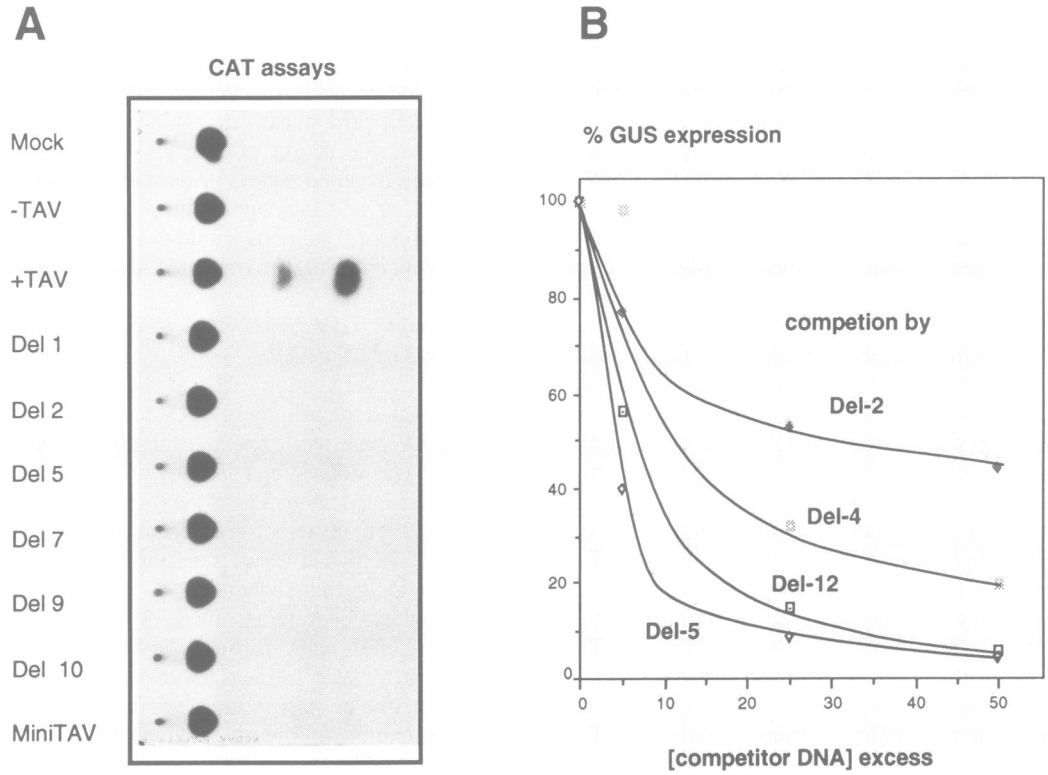

Fig. 6. Transactivation (A) and competition (B) experiments in *O.violaceus* protoplasts. (A) The dicistronic reporter plasmid, pCAT2, the transactivator plasmids pHELP7 or several pDel derivatives (names on the right) were cotransfected by electroporation in protoplasts from *O.violaceus* cell cultures. Typical CAT assays are shown. (B) Competition experiments were performed as described in Figure 5. Competitor plasmids were pDel-12, pDel-2, pDel-4 and pDel-5 at 5 ×, 25 × and 50 × excess concentration compared to pHELP7.

1987). This single purification step yielded TAV protein with 80–90% purity. The protein was solubilized in 8 M urea and renatured by stepwise dialysis. Purity and integrity of TAV was checked by staining of SDS–PAGE and by immunoblotting with an anti-VI antiserum.

This TAV preparation was used in gel-shift experiments (Figure 7). Three types of RNA probe were designed to assay the specificity of the TAV–RNA interaction: a 380 nt CaMV-specific probe [consisting of part of the leader region preceding ORF VII, a shortened ORF VII (by an in-frame deletion) and the intercistronic ORF VII-I region], a 101 nt nonspecific probe derived from the pGEM2 vector and a probe similar in length to the CaMV probe (380 nt) derived from anticlockwise transcription of CaMV ORF II. As a protein control, we used a total extract of uninduced *E.coli* cells and a CaMV protease preparation formed from BIB and renatured in parallel to the TAV BIB. The latter control ensured that an observed RNA gel-shift did not result from interactions with an *E.coli* protein that might co-precipitate with the aggregates of foreign protein. Samples containing RNA probes and increasing amounts of solubilized TAV protein or control protein preparations were incubated and loaded onto a native polyacrylamide gel. The results show that all three RNA probes were shifted by the action of the TAV protein preparation but not by the two control preparations (Figure 7, not shown for the pGEM vector probe). Thus, gel-shifting by TAV was nonspecific in respect to the probe sequence. The protein–RNA complex formation was cooperative and occurred even at a low concentration of TAV protein (5 ng/μl). At higher concentrations (25 and 50 ng/μl), the complexes were trapped in the gel slot, while part of the RNA moved to the position of the free probe. No aggregates of TAV protein were observed in solutions of up to 10 μg/μl RNA-free protein (not shown). The protein–RNA interactions seemed to trigger protein–protein aggregation. The RNA binding was competed by poly(U) and M13 ssRNA, but not by tRNA, poly(I)–poly(C) or native salmon sperm DNA (not shown), indicating that the interaction may be specific for single-stranded nucleic acid. Since the MiniTAV protein did not form BIBs in *E.coli* and, therefore, could not be purified easily as such, we provided its C-terminus with an affinity tag consisting of six histidines. The protein was expressed in *E.coli* and purified based on the selective affinity of adjacent *his* residues for a Ni^{2+}–metal chelate adsorbant (Ni-NTA resin). The purified MiniTAV-6 × His protein did not cause RNA gel shifts (Figure 7C), showing that MiniTAV does not contain major RNA-binding domains.

North-western experiments using TAV and TAV derivatives were performed in order to define more precisely the RNA-binding domain of TAV; the mobility of TAV as an antigen and the presumptive RNA-binding protein were compared (Figure 8). Crude *E.coli* control extract yielded an intense pattern of bacterium-derived RNA-binding proteins (CE, Figure 8, panel I); TAV and some of its derivatives migrate in a window of this pattern (as seen on the immunoblot in Figure 8, panel IB) and hence could be assayed in the crude extract. Other derivatives were purified as bacterial inclusion bodies and thereby freed from most of the bacterial background bands. Del-7 protein, which does not form inclusion bodies, was assayed from a semi-purified extract with relatively low background. TAV, Del-2, Del-4, Del-5 and Del-10 clearly showed RNA-binding capacity but Del-7, -9 and -11 did not (Figure 8, panels IC and IIB). The

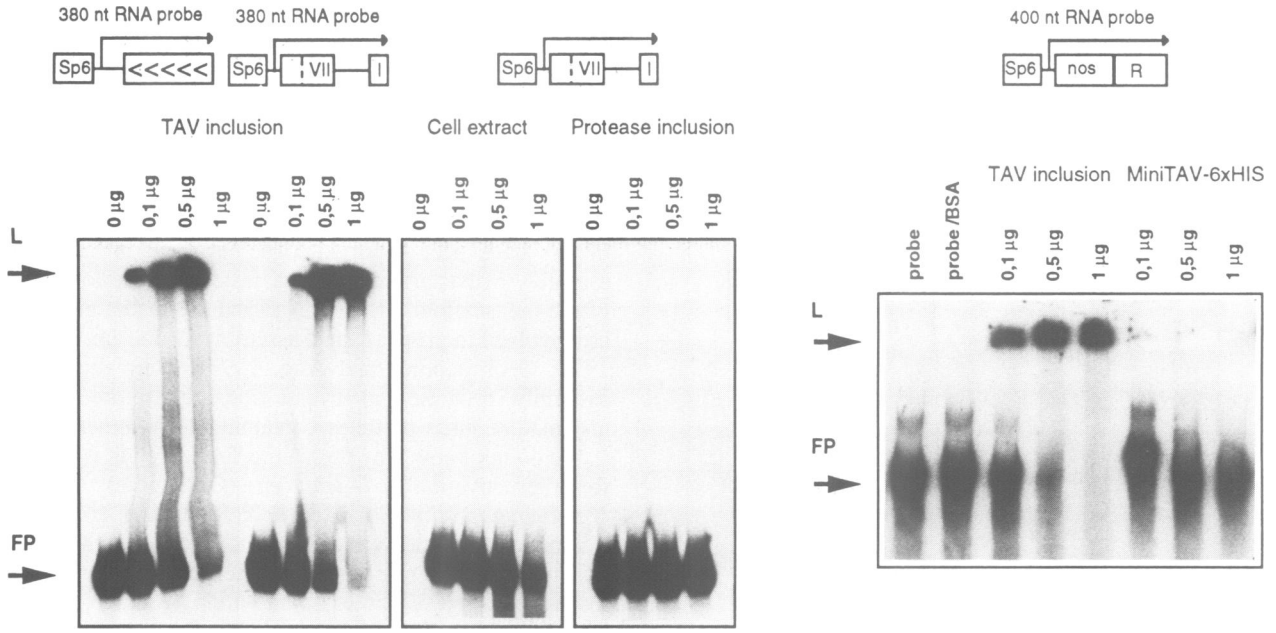

Fig. 7. RNA binding of TAV and MiniTAV produced in *E.coli* and analyzed by gel-shift experiments. RNA probes (shown at the top of the figure) were incubated as described in Materials and methods with increasing amounts (indicated on the figure) of refolded TAV protein from a bacterial inclusion body (BIB), from cell extracts and from protease BIB, or with purified MiniTAV-6 × HIS protein. Control lanes with the probe alone or the probe in the incubation mix with bovine serum albumin (BSA) are indicated. The positions of the slot (L) and of the free probe (FP) are indicated with arrows.

comparison of Del-5 and Del-7 revealed a protein domain (a) between amino acids 242 and 305 which, if present, confers RNA-binding properties to TAV. Comparison of Del-10 with Del-7 and Del-11 defined another domain (b) between residues 346 and 378. We do not understand why Del-9 does not bind RNA although it contains the second domain, and assume that this binding domain is sterically occluded. The domains identified may be involved directly in RNA binding, but they could also be involved in properly presenting the binding domain. The domain 'a' is located within a region highly conserved in caulimoviruses. Amino acids 294–305 within this domain have the sequence AAGLI, which is related to the LAGLV and LAGLI pentapeptides from the TMV p30 movement protein (Citovsky *et al.*, 1990) and from yeast intron-encoded mitochondrial protein (Zimmern, 1983), respectively. In both cases, this sequence was considered to be involved in RNA-binding domains, mediating cell–cell transport of TMV RNA in the one case and splicing in the other. Neither of the two binding domains overlaps the sequence defined by the MiniTAV. We conclude, therefore, that RNA binding is not a condition *sine qua non* for transactivation, although it might well stimulate it.

Discussion

The CaMV ORF VI protein in its function as a transactivator enhances translation from CaMV RNA and from dicistronic reporter plasmids both in protoplasts (Bonneville *et al.*, 1989; Gowda *et al.*, 1989) and in transgenic plants (Zijlstra and Hohn, 1992). Considering the electron microscope observations that polysomes accumulate at the surface of the CaMV inclusion bodies, which consist mainly of TAV (Shepherd, 1976), and the fact that TAV copurifies with polysomes (A.Himmelbach, unpublished observation), it is our hypothesis that the transactivator interacts directly with the translational machinery, rather than promoting the expression of other factors directly responsible for the process.

Here we report that a small portion of the TAV protein, MiniTAV, is sufficient to transactivate translation of a dicistronic reporter plasmid in *N.plumbaginifolia* (Solanaceae) mesophyll protoplasts. This shows that the MiniTAV domain contains the active center of the transactivator, while the rest of the protein has other functions. Similar levels of transactivation can be reached with MiniTAV and with TAV, but ~100-fold higher plasmid DNA concentrations are required for MiniTAV. This suggests that the role of some of the protein domains outside of the MiniTAV domain is to activate the MiniTAV domain or to concentrate TAV molecules at their site of action. For example, the RNA-binding domains directly or indirectly responsible for RNA binding which we identified in the C-terminal half of TAV may help to concentrate the transactivator around the mRNA and the ribosomes, promoting its interaction with the translational machinery. The cooperativity of RNA binding should amplify the concentration effect. This cooperativity also suggests the existence of TAV–TAV interaction sites which come into action, perhaps by an allosteric effect, after the binding of neighboring TAV molecules to RNA has occurred. If such complexes are formed with a defective TAV derivative, inactive complexes may result and lead to the competitive inhibition that we observed. Other domains of the TAV protein may bind cofactors which are limited in reticulocyte lysates or wheat germ extracts, explaining why transactivation can be observed in plant protoplasts but not *in vitro* (Gordon *et al.*, 1988). Experiments on the supplemention of *in vitro* systems with plant cell extracts should help in testing this hypothesis.

As mentioned above, TAV is also the major component of the large viral inclusion bodies or viroplasms discernible even by light microscopy (Shepherd, 1976). These inclusion

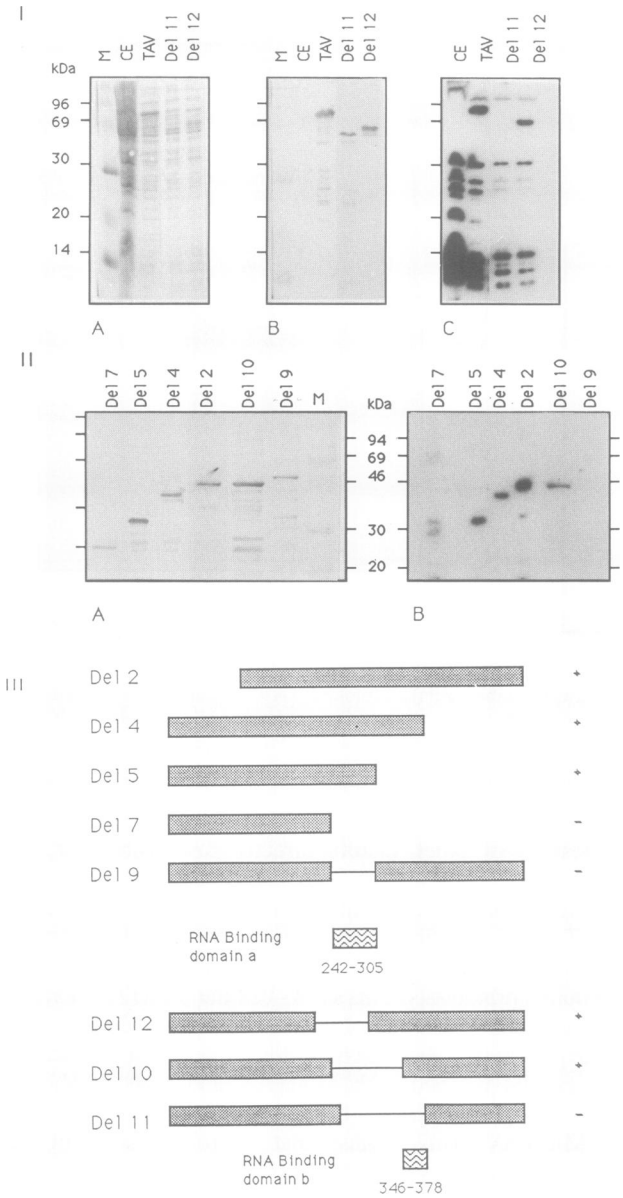

Fig. 8. RNA binding of TAV and derivative proteins produced in *E.coli* and analyzed by North-western blots. (**I**) Whole cell extracts. The proteins were electrophoresed on 10% SDS−PAGE and transferred onto nitrocellulose filters. Proteins were revealed by amido black staining (A), by immunoblotting with anti-VI antiserum (B) and by probing with labelled RNA after blocking with Denhardt's solution containing SBB buffer and renaturation of the blotted proteins after an overnight incubation in SBB buffer (C). Molecular weight markers (M) are indicated. (**II**) Inclusion body preparations. The solubilized proteins (1 μg) were electrophoresed on 12% SDS−PAGE and transferred to nitrocellulose filters. Proteins were revealed by immunoblotting with anti-VI antiserum (A) and by probing with labelled RNA as above after blocking with Denhardt's solution (B). Molecular weight markers (M) are indicated. (**III**) Schematic representation of the proteins analyzed and of the RNA-binding domains deduced from the experiment.

bodies were thought to be involved in the occlusion of proteins produced by CaMV RNA (deZoeten *et al.*, 1989), in virus assembly (Shepherd, 1976) and in reverse transcription (Pfeiffer and Hohn, 1983; Mazzolini *et al.*, 1985, 1989). Such functions may all be associated with specific domains of the TAV protein and may have a general purpose, i.e. to confine cytoplasmic virus activity to a specific compartment, thereby minimizing cytotoxic effects and interaction with cellular defense mechanisms. The following scenario would explain how the various functions are connected: after the initial interactions of TAV with CaMV RNA, polycistronic translation on the one hand and TAV aggregation on the other are induced and the nascent translation products are transported to the interior of these aggregates, where particles consisting of capsid protein, virus RNA and Pol polyprotein assemble. Virus and host-coding factors have been reported to be involved in the proper assembly of bacteriophages, for instance bacteriophage λ, where the 'scaffold' protein pNu3 and the chaperonin pgroE are involved in proper capsid assembly (Hohn and Katsura, 1977; Georgopoulos and Ang, 1990). Following proper assembly, protease and reverse transcriptase are activated and mature virus particles containing DNA emerge.

We do not know why the whole TAV protein is required for transactivation in *O.violaceus* (Cruciferae) but not in *N.plumbaginifolia* (Solanaceae) cell culture protoplasts. Various possibilities were proposed in the Results section but, whatever the reason, for our purpose it was important that at least one system exists in which MiniTAV is active and can be studied. The activity of MiniTAV in *N.plumbaginifolia* suggests that the other domains are not directly implicated in the molecular mechanism of translational transactivation and may have other functions. Moreover, the fact that none of the mutants is accepted in *O.violaceus* confirms that TAV is a key for host specificity. The virus infection cycle can be directly controlled through the transactivation function of TAV as virus expression in the host cell needs a strictly conserved TAV protein.

Since TAV transactivates translation not only from virus-related RNAs (Bonneville *et al.*, 1989; Gowda *et al.*, 1989; Fütterer *et al.*, 1990a; Scholthof *et al.*, 1992) but also from dicistronic reporter plasmids free of CaMV sequences (Fütterer and Hohn, 1992), it is likely that it can also enhance translation from an authentic plant mRNA containing upstream ORFs. This would result in overexpression of the gene in question, and could be one of the reasons for the abnormal phenotypes seen in TAV-transgenic non-host plants (Baughman *et al.*, 1988; Takahashi *et al.*, 1989; Balàsz, 1990; Goldberg *et al.*, 1991) and e.g. *A.thaliana* host plants. The stunting of *A.thaliana*, enlargement of its rosettes and delayed flowering suggest that a developmentally controlled gene is affected (Zijlstra and Hohn, 1992).

Materials and methods

Plasmids

Plasmid pDH51 (Pietrzack *et al.*, 1986), the basic vector for most of our constructs, consists of the CaMV 35S promoter, a multiple cloning site and the CaMV polyadenylator cloned into pUC18. pKG1 (Gordon *et al.*, 1992) is a derivative of pDH51 containing within the multiple cloning site an ATG translation start codon with optimal context (AAACATGGCG; Lütcke *et al.*, 1987) followed directly by an *Xho*I cloning site.

Plasmids pCAT2, pCAT3, pBiGUS, pMonoGUS and pHELP7 with the pDH51 background have been described (Bonneville *et al.*, 1989).

Plasmid pDel-1 (Figure 9) was constructed by cloning the *Hin*dIII−*Xba*I fragment from pHELP7 into pKG1. Plasmids pDel-2 and pDel-3 were produced from pDel-1 by deleting the internal *Sal*I−*Eco*RI and *Sal*I−*Mlu*I fragments, respectively, and filling in the restriction ends before ligation.

Plasmids pDel-4, -5, -6 and -8 were produced by insertion of a universal translation termination linker [(*Afl*II)-CCT*TAAGCTAGCCTAG*GACAG-GATCC-(*Bam*HI)] into the polished second *Nco*I, *Ava*I, first *Nco*I and *Stu*I restriction sites of pHELP7, respectively. Versions of these plasmids with the TAV sequences after the termination linker also physically removed were obtained by deleting the internal *Bam*HI fragment between the sites

Fig. 9. Restriction map of the TAV ORF and surrounding sequences in pHELP7 and pDel-1. The restriction sites relevant for the plasmid construction described are shown.

at the end of the linker and after the original TAV stop codon. In plasmid pDel-7, obtained by deleting the internal *Bgl*II−*Bam*HI fragment of pHELP7, the TAV frame switches to another phase which stops two amino acids after the site of the switch.

Plasmids pDel-9 and pDel-12 were obtained by deleting the internal *Nco*I and *Stu*I fragments, respectively, of pHELP7. Plasmids pDel-10 and pDel-11 were obtained by deleting the internal *Bgl*II−*Ava*I and *Bgl*II−*Sca*I fragments, respectively, of pHELP7, the restriction ends being filled in with Klenow polymerase before ligation.

Plasmid producing MiniTAV and other TAV derivatives with two deletions (e.g. pDel-12:4 and pDel-12:5) were prepared by combining the plasmids containing the single deletions through the central *Mlu*I site of the TAV ORF.

Plasmid pDHTZ is pDH51 with the pUC18 vector background changed to that of vector pTZ19u (Mead *et al.*, 1986). The *Eco*RV−*Xba*I fragment of pHELP7 containing the TAV ORF was cloned into pDHTZ, and single-stranded DNA produced from the resulting construct was used for oligonucleotide-directed mutagenensis for the deletion and the amino acid exchanges described. The Amersham *in vitro* mutagenesis kit was used.

For expression in *E.coli*, the *Hind*III−*Xba*I TAV fragment from pHELP7 was cloned into the pDS56/RBSII-2 vector (Bujard *et al.*, 1987) to yield pDSTAV. Plasmids expressing TAV deletion mutants in *E.coli* were produced by exchanging the *Mlu*I−*Xba*I restriction fragment containing the C-terminal portion of TAV in pDSTAV with the *Mlu*I−*Xba*I restriction fragment of the mutant in question.

Transient expression

Samples of 6×10^5 *N.plumbaginifolia* leaf protoplasts were used for polyethylene glycol (PEG) transfection and samples of 2×10^6 *O.violaceus* cell suspension protoplasts for transfection by electroporation (Fromm *et al.*, 1986) with DNA as described by Bonneville *et al.*, (1989). Culture conditions and protoplast preparation were as described previously (Fütterer *et al.*, 1989, 1990b). Routinely, 5 μg dicistronic reporter plasmid, 5 μg helper plasmid and 0.5 μg monocistronic standardization plasmid, all circular, were used in each assay with *N.plumbaginifolia* protoplasts. When pBiGUS was the dicistronic reporter plasmid, pCAT3 (Bonneville *et al.*, 1989) was used as the standardization plasmid; when pCAT2 was the dicistronic reporter plasmid, pMonoGUS was used for standardization. For *O.violaceus* protoplasts, the amounts were similar, but 1 μg of standardization plasmid was used. After 18−24 h incubation at 22°C in the dark, protoplasts were harvested and soluble extracts were prepared. CAT and GUS activities were determined as described previously (Gorman *et al.*, 1982; Jefferson *et al.*, 1987; Bonneville *et al.*, 1989; Fütterer *et al.*, 1989).

Competition assays

For competition experiments in *N.plumbaginifolia*, protoplasts were transfected with 5 μg dicistronic reporter plasmid, 0.2 μg transactivator plasmid, competitor plasmid in increasing amounts (0.2−10 μg) and 0.5 μg monocistronic normalization plasmid. Similar mixtures were used for competition experiments in *O.violaceus*, but with 1 μg transactivator plasmid and 1 μg internal control plasmid. For any given experiment, the total amount of transfected DNA in each sample was adjusted to the same value by adding sheared calf thymus or salmon sperm DNA. CAT and GUS activities were measured as above. For quantitative determination of CAT activity, the bands of chloramphenicol and 3-acetyl chloramphenicol were excised from the TLC plate and their radioactivity determined by scintillation counting. GUS activities were assayed in microtiter plates in 250 μl samples containing 1 mM *p*-nitrophenyl glucuronide (Jefferson *et al.*, 1987). Aliquots of 50 μl were removed at four successive time points and the appearance of *p*-nitrophenol was followed spectrophotometrically at 405 nm. Dicistronic expression was corrected for fluctuation in transfection efficiency by considering the internal monocistronic standard.

Protein production in E.coli

E.coli M15 cells containing the km^R pDMI.1 plasmid (Certa *et al.*, 1986) were transformed with pDSTAV or its deletion derivatives. Growth and IPTG induction of TAV expression from the T5 *lac* promoter were as described by Torruella *et al.* (1989). BIBs were prepared and purified as described previously (Nagai and Thøgersen, 1987), dissolved in 8 M urea in TBS buffer (50 mM Tris−HCl pH 7.5, 150 mM NaCl), diluted with 1 vol. of TBS buffer and subsequently dialysed against 3.5, 3, 2, 1 and 0.5 M urea and then 30% glycerol, all in TBS. Protein concentrations were determined using the Bradford assay in microtiter plates.

Construction of the plasmid that expresses MiniTAV with a 6 × His tag

MiniTAV DNA was amplified by PCR and cloned into the *Bam*HI and *Bgl*II sites of plasmid pQE-17 (pDS-56 derivative, QAexpress SYSTEM, QIAGEN), thereby replacing the dihydrofolate reductase by MiniTAV and fusing the C-terminus of MiniTAV to six adjacent histidine residues (6 × His tag). The construct was verified by sequencing.

Expression and purification of MiniTAV-6 × His

The protocol was essentially as described by the manufacturer and Hochuli *et al.* (1988). Briefly, expression of a MiniTAV-6 × His in M15 cells was induced with IPTG for 3 h. Cells were harvested and lysed in 6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris pH 8.0. The clear lysate was pumped onto the Ni^{2+}−NTA resin (Qiagen) equilibrated with lysis buffer. After washing with 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris pH 8, the column was eluted by decreasing the pH stepwise. The pH 5.9 eluate contained pure MiniTAV-6 × His protein, which was refolded and renatured as described above and used for gel-shift experiments.

RNA probes

RNA probes were produced by *in vitro* transcription of linearized template DNA with SP6 polymerase in the presence of [^{32}P]GTP (80 mCi/pmol, 25 mM). Probes were eluted from a preparative polyacrylamide gel and directly used in North-western or gel-shift experiments.

North-western assays

After SDS−PAGE and electrotransfer of proteins to nitrocellulose sheets, unreacted sites on the membranes were blocked for 30 min at room temperature with standard binding buffer (SBB; 10 mM Tris−HCl pH 7, 1 mM EDTA, 50 mM NaCl) containing 1 × Denhardt's solution (Maniatis *et al.*, 1982). Protein refolding was performed by incubating the filter in 1 × SBB at 4°C overnight. The blotted proteins were probed by incubation with 10^4-10^5 c.p.m./ml of the ^{32}P-labelled RNA probe for 1 h at room temperature. Unbound RNA was removed by three cycles of washing with SBB. Filters were air dried and autoradiographed.

RNA gel-shift assays

The indicated amounts of protein extract or purified BIBs were incubated for 1 h at room temperature in 20 μl sample buffer (25 mM HEPES pH 7, 2 mM DTT, 8 mM $MgCl_2$, 4 mM spermidine, 50 mM NaCl, 10% glycerol); in addition each assay contained 12.5 units RNasin, 1 μg tRNA and/or 1−5 μg poly(I)-poly(C) with 10^4-10^5 c.p.m. of the RNA probe in question. Samples were then separated on a 4% native polyacrylamide gel in low ionic strength electrophoresis buffer (8 mM Tris−HCl pH 7.9, 3.5 mM sodium acetate pH 7.9, 1 mM EDTA) with constant recirculation of the buffer. The dried gels were autoradiographed.

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